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Simple In Vivo Production and Storage Methods for *Steinernema carpocapsae* Infective Juveniles[1]

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Abstract: Methods are described for standardized in vivo production, rapid harvest, and storage, in a concentrated form, of infective juveniles of the entomopathogenic nematode, *Steinernema carpocapsae* Mexican strain Kapow selection. Nematodes were stored in nematode wool configurations, consisting of mats of intertwined infective juveniles. Freshly harvested nematodes are readily available in adequate quantities for laboratory and small-scale field evaluations as well as cottage industry production.

Key words: Bioassay, *Galleria mellonella,* Kapow selection, Mexican strain, nematode, nematode wool, *Steinernema carpocapsae.*

Economical in vitro rearing methods (3,12), are appropriate for commercial and large-scale laboratory production of steinernematid nematodes. In vivo rearing methods (4,5,8,9,11–13) are required for initial isolation and production of new nematode accessions, and species or strains not available from commercial sources. The need for a standardized in vivo production and storage procedure to minimize test variability and provide a readily available source of freshly harvested infective juveniles (IJ) led us to investigate our production and storage methodology.

MATERIALS AND METHODS

Host larvae of the greater wax moth, *Galleria mellonella* (L.), were reared by modifying the methods of Poinar (11). The Mexican strain of *Steinernema carpocapsae* Weiser was used in this study. To insure rapid production of IJ and good biological activity and virulence, the population was maintained by Kapow selection procedure in which first-migrating, trapped IJ are used for initiating the next generation (1).

Galleria mellonella mortality was evaluated for *S. carpocapsae* IJ production with a time versus concentration bioassay (LT_{50}). Recently harvested IJ were counted by pipetting 0.02-ml drops of IJ–water suspension on the bottom of a 100- × 15-mm plastic petri dish. The drops were overlaid with 9-cm diameter filter paper, and deionized water was added to give a total volume of 1 ml per dish. The dish was covered with a lid and allowed to equilibrate for 1 hour, then 10 *G. mellonella* larvae (selected for large size, ca. 0.26 g) were added to each petri dish. The test consisted of five replications of nematode concentrations of 0, 5, 15, and 50 per *G. mellonella* larva (i.e., 0, 50, 150, and 500 nematodes/dish). The dishes were incubated at 25 C and 45% RH. Beginning 16 hours after initial exposure to the nematodes, larval mortality was determined hourly for 15 hours and at 48 and 72 hours.

Log/probit models were used to fit both concentration/mortality and time/mortality responses. Means and standard errors for lethal median concentrations (LC_{50}) and lethal median times (LT_{50}) are computed from the four tests in time.

For rearing, an "exposure" dish (petri dish: 150 × 25 mm) was used with a 150-mm Whatman qualitative #1 filter paper placed in it. Fifty *G. mellonella* larvae (ca. 12 g) were added and an additional filter paper was placed over these larvae to reduce webbing and facilitate the removal of host

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cadavers. A water–nematode suspension (2,500 IJ per dish) was added to the top filter paper and total volume of water was brought up to 3 ml per dish. The dish was then covered with a lid with five 3-mm-d ventilation holes drilled in the top and inserted into a 19- × 33-cm Tower dualpeel® sterilization pouch; the end of the pouch was folded three times and secured with a paper clip. The pouch was freely permeable to O_2, provided visual assessment of nematode production, and reduced contamination and water loss. The exposure dish was stored at room temperature (25 ± 3 C) for 48 hours.

A "production" dish was prepared by adding 20 ml of thinned plaster of Paris mixture (1 water: 1 plaster, v/v) with a 10-ml S/P varipet pipetter to the bottom of a 100- × 15-mm plastic petri dish and allowing it to air dry. When rewetted, the plaster (6) provides a subsurface water reservoir that provides humidity as well as a margin of splash protection, absorbing ca. 10 ml of water. It also maintains a relatively dry plaster–host interface suitable for nematode reproduction and the migration of newly produced IJ. Nematode-infected *G. mellonella* cadavers were transferred from the exposure dish to this "production dish" (Fig. 1A), which was then placed inside a 150- × 25-mm plastic dish containing 10 ml deionized water. Both uncovered dishes were inserted into a sterilization pouch, which was closed (Fig. 1B).

Nematodes were harvested by transferring the nematode suspension at daily intervals from the production dish into an aerated 1-liter flask, rinsing out residual nematodes and refilling the dish for the next IJ migration. Production dishes were replicated five times with three tests in time. Infective juveniles were harvested daily, except for the first test, which was harvested bi-daily. Harvested nematode suspensions were volumetrically measured, counted (10), and concentrated for storage. Nematodes can be concentrated by applying negative pressure to a medium-grade aquarium air stone using a water trap to capture the water filtrates for disposal. Positive air flow can be used to unclog the air stone.

A "nematode wool" concentrate was produced by substituting a reusable nylon mesh with 10.0-μm openings (Spectra/Mesh) for the fiberglass filter in a vacuum-Leramie-Buchner funnel method (10) (Fig. 1C). Nematodes can then be stimulated to intertwine forming a mat or "nematode wool" configuration by continuing the vacuum pressure negative air flow for an additional 2 minutes after the initial free water removal. The wool was removed from the nylon screen (Fig. 1D), rolled up, and inserted into a 275-ml Corning tissue culture flask containing 20 ml dry plaster of Paris mixture on the upper and lower sides. Two ml of deionized water was then added to the plaster of Paris to maintain a high RH. The container was closed with a Corning phenolic style cap in the open position, one-half turn from closed, and placed at 7 C to inactivate the nematodes while in the wool configuration.

The weight of nematode wool containing 1.5×10^6 IJ (five observation, three replications), 5×10^6 IJ (5 observations, 6 replications), and 15×10^6 IJ (9 observations) was determined.

Results and Discussion

For the Kapow selection *S. carpocapsae* Mexican strain used in this study, the mean LC_{50} was 3.25 ± 0.83 (S.E.) nematodes/larva, with 95% confidence limits of 0.62 to 5.88. The mean LT_{50} at the highest concentration (50/larva) was 27.2 ± 1.47 hours (S.E.), with 95% confidence limits of 22.5 to 31.9 hours (Table 1). Means plus standard error provide the criterion for our standard quality control bioassay. To fall within Kapow IJ quality standards, wax moth larval mortality must reach or exceed 50% at both 72 hours, 41 IJ/dish, and 29 hours, 500 IJ/dish.

Production of the IJ from *G. mellonella* larvae (25 ± 3 C) began at 7–9 days after IJ-host exposure. Average total IJ production over a 16-day period, starting after

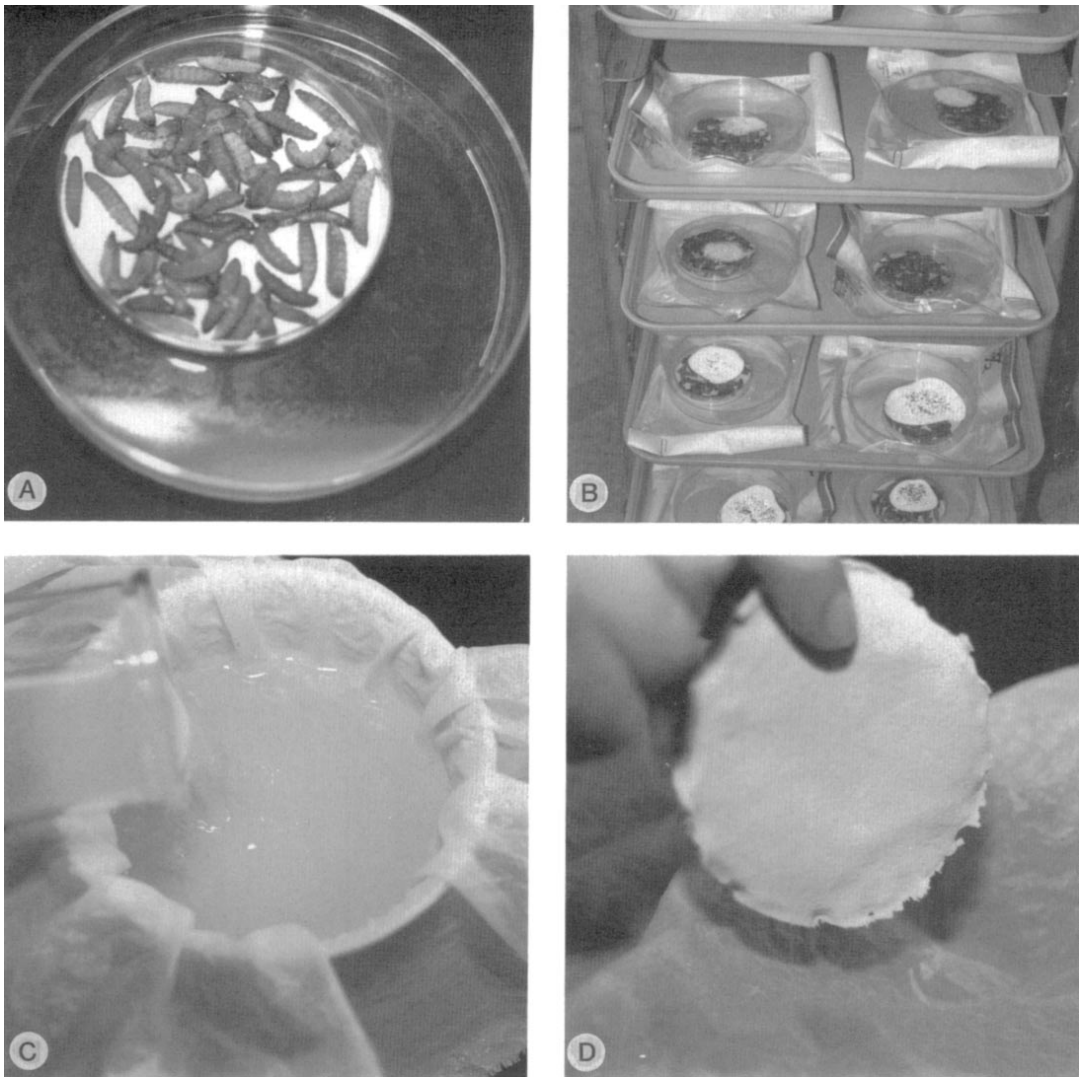

FIG. 1. Production, harvest, and storage method. A) Production dish with emerged nematodes in water trap. B) Nematode production shelved on mobile pastry rack. C) Harvested, bulked, and counted nematode suspension added to nylon screened Buchner funnel for concentration. D) After water removal, nematode wool can be lifted from mesh for storage.

emergence from the host was 1.9×10^5 IJ per host larva (8.2×10^5/g of host or 9.9×10^6 per production dish). Daily harvests of IJ production peaked at day 2 and 10 and declined at day 14. Daily harvests from tests 2 and 3 were combined to two-day values comparable to test 1 (Fig. 2).

Regression analysis of the nematode wool showed that the IJ counts of 1.5, 5.0, and 15×10^6 were very highly correlated with the IJ weight; $r^2 = .98$ ($N = 39$). Total mean gram weight per million nematodes was 0.21 g/million, SE = .0050 (9 degrees of freedom—based on variance among tests). Differences between IJ treatments were not significant by ANOVA, $P = .52$.

Steinernema carpocapsae IJ reared on *G. mellonella* larvae have been stored as nematode wool at ca. 7 C up to 6 months using this method. Nematode mortality was not observed ($N = 50$). Stored IJ exceeded the LC_{50}-LT_{50} quality control bioassay standards for Kapow selected IJ.

TABLE 1. Predicted percent mortality of last instar *Galleria mellonella* larvae exposed to three concentrations of *Steinernema carpocapsae* infective juveniles.

Concentration per larva	Hours	Predicted % mortality	95% C.I.
50	24	29.5	12.0, 50.9
50	48	99.1	93.4, 99.5
50	72	100.1	99.9, 100.0
15	24	14.7	05.1, 28.3
15	48	88.9	66.7, 99.6
15	72	98.9	93.7, 99.8
5	24	04.9	03.8, 06.2
5	48	54.9	31.4, 77.2
5	72	60.6	44.7, 75.4

A concentration of 50 nematodes per *G. mellonella* larva, 2,500/dish, is considered as the appropriate production exposure concentration for both rapid host mortality and assessment of IJ virulence 48 hours after exposure. A combined host mortality of less than 93.4% C.I. (4 or more surviving larvae from 50 larvae per treatment), would indicate a substandard nematode inoculum.

Reported IJ production has varied depending on host size, host and nematode species, and rearing method. Although Dutky (8) reported lower IJ production per *G. mellonella* larva, 1.6×10^5 compared to our 1.9×10^5, his smaller larvae produced more IJ per g of host (11.1×10^5 compared to our 8.2×10^5). Nonproductive insect cadavers reduce overall IJ production. Blinova and Ivanova (4) reported that only 71.1% of the parasitized *G. mellonella* larvae in their cultures were IJ-productive. In contrast, our production method using disease-free colony *G. mellonella* provides 98.6% IJ-productive larvae.

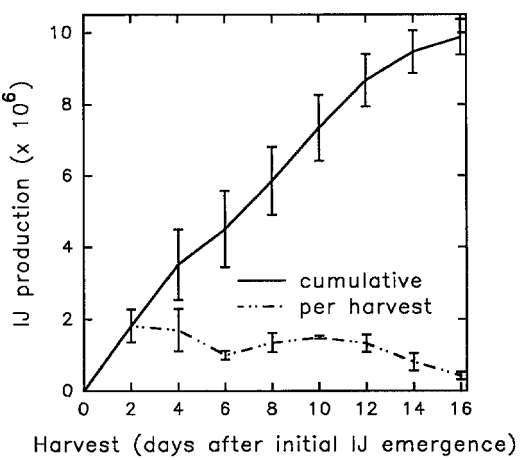

FIG. 2. Mean bi-daily and cumulative harvest of *S. carpocapsae* Kapow selection infective juveniles per dish with standard error bars (5 production dishes, 50 larvae, 12 g per dish, 3 replications in time).

Other entomopathogenic nematodes we have reared with this method include *S. carpocapsae,* All and UK strains, *S. feltiae* (bibionis), *Heterorhabditis bacteriophora* (HP88), and *H. bacteriophora* Australian strain.

This rearing method does not require the use of incubators, autoclaves, or toxic disinfectants and provides an aerobic humid environment for optimal nematode production. It minimizes quality control needs by maintaining the phase one symbiotic bacterium (2); reduces unwanted insect, fungal, and bacterial contaminants by its closed pouch environment; and generates only infective juveniles by containing adults and non-IJ larval stages within the production petri dish. Infectives free of other unwanted nematode stages and food contaminants can be rapidly harvested and concentrated into a nematode wool configuration.

This tendency of IJ to form a wool in response to free water removal may be similar to the preanhydrobiotic coiling induced in three soil nematode genera by Demeure et al. (7). Storing the IJ as nematode wool allowed for O_2 distribution and eliminated the need for the commercially used dispersant materials such as polyurethane foam, charcoal, or vermiculite. Nematode wool provides a concentrated IJ-only product; the number of IJ can be estimated by weight. It is easily stored and transported to an application site. Nematodes can be added directly to sprayer tank water without the need to separate them from storage and shipping formulations.

This in vivo production technique provides a ready source of nematodes in adequate quantities for laboratory and small-scale field investigations and cottage indus-

try production at an estimated cost, excluding labor and overhead, of $0.15 per million.

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